Localization of the Control Region for Expression of Exotoxin A in *Pseudomonas aeruginosa*

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The 2,760-base-pair (bp) *PstI-EcoRI* segment of the chromosome of *Pseudomonas aeruginosa* PA103 which carries the exotoxin A structural gene was expressed from an internal promoter when cloned in a pUC9 derivative and transformed into a nontoxigenic mutant of *P. aeruginosa* PA01. The unique terminal EcoRI site was deleted, and a new EcoRI site was substituted for a *PvuI* site located 107 bp 5' to the transcription initiation site. Following *EcoRI* cleavage, *Bal31* deletions were generated from this site, and an *EcoRI* linker sequence, GGAATTCC, was inserted in place of the deleted DNA. Mutants with deletions located 73 bp or more upstream of the transcription initiation site retained normal expression, whereas in mutants with deletions extending into the region 69 bp or less upstream of this site, exotoxin synthesis was greatly reduced. From a *KpnI* site located 473 bp 3' to the transcription initiation site, a similar series of *Bal31* deletion mutants were generated in which the inserted *EcoRI* linker sequence was located within the same 72-bp region. Pairs of mutants from the two deletion series were identified in which the *EcoRI* linker was located at the same sequence, and these mutant pairs were ligated to derive a series of constructs in which the *EcoRI* linker sequence GGAATTCC was substituted for an 8-bp sequence within the 72-bp region. Some of these linker-substituted mutants showed greatly reduced exotoxin A synthesis. The results are consistent with a binding site for a positive activator contiguous with the binding site for an RNA polymerase.

We and others have reported that expression of the *Pseudomonas aeruginosa* exotoxin A (*toxA*) structural gene in *Escherichia coli* requires the presence of an external upstream promoter (9, 20). In contrast, when the cloned gene was transformed into MAM4, a *recA*, nontoxigenic mutant of *P. aeruginosa* PA01, we found that exotoxin was synthesized and secreted into the culture supernatant (8). This expression was shown to be due to a single transcript initiated 88 base pairs (bp) upstream of the initiating codon (Fig. 1). Moreover, when iron was present in excess of 0.1 mM, which inhibits the expression of exotoxin A in *P. aeruginosa* (4), we detected no transcription (8). In the sequence of 80 or more nucleotides upstream of the initiation site, no homology of the *E. coli* 70 bp promoter consensus sequence (11) could be found (Fig. 2), even though it is known that other *Pseudomonas* genes, such as that for β-lactamase, are transcribed from promoters whose sequences closely resemble the *E. coli* consensus sequence (7).

Other experiments (12, 23) have established that the product of a second gene, termed *toxR*, is required for expression of *toxA* and that, like *toxA*, transcription of *toxR* is inhibited by iron (13). Thus, a simple model for the regulation of *toxA* would be that its transcription requires the binding of the positive activator *toxR* product, and it is therefore indirectly regulated by iron through the repression of *toxR* transcription.

One of the goals of this research is to identify the nucleotide sequences required for exotoxin A expression in *P. aeruginosa* and to further clarify its regulation by iron by the construction and characterization of "linker-scanning" mutants (19).


MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strains HB101 (hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 met-l supE44 λ- F- [6]) and AA102 (recA pro thi supE endA hsdRk (gal-chID-plg-attB)) (1) and MAM4, a recA102 *met-28 toxA* mutant of *Pseudomonas aeruginosa* PA01 (8, 20) were used as hosts for the construction and expression of the cloned exotoxin A gene segment. MAM4 was isolated by nitrosoguanidine treatment from PAO286, a *met trp* derivative of the genetic progenitor strain PA01. It was concluded to have a mutation in or near the *toxA* structural gene by linkage mapping and its ability to be complemented by *pRC362* (8).

The vector *pRC357* was constructed from *pUC9* (22) by insertion of a 1.85-kilobase (kb) *PstI* segment from *pRO1614*, which permits replication in *Pseudomonas* hosts (21), and subsequent deletion of the *PstI* site distal to the multiple cloning site of *pUC9* (8). *pRC360* was constructed by inserting into *pUC9* the 2,760-bp *PstI-EcoRI* chromosomal segment of *P. aeruginosa* PA103 (15) that carries the exotoxin A gene, and *pRC362* carries the same *PstI-EcoRI* segment inserted into *pRC357* (8). *pUC4-KISS* (Pharmacia, Inc.) was the source of a 1.3-kb kanamycin resistance (Km') cassette (2), excised by *EcoRI* digestion. The level of expression of exotoxin A by *pRC362* in MAM4 (about 6 μg per OD600 unit) was very similar to that found when *pRC362* was expressed in PA01 when the presence of an active chromosomal *toxA* gene in PAO1 and not in MAM4 is taken into account.

Media and biologicals. The media used were *Oxoid* No. 2 nutrient broth (NB) and nutrient agar (NA) and tryptic soy broth dialyzed (TSBD) as described previously (8), except that TSBD was defferrerated to about 1 μM by using Chelex 100 (Bio-Rad Laboratories) (14). Solid TSBD was prepared by the addition of 0.8% agarose (SeaKem). Where appropriate,
FIG. 1. Details of the exotoxin A gene fragment cloned in pRC362. The 2,760-bp PstI-EcoRI chromosomal fragment of P. aeruginosa PA103 was cloned in a pUC9 plasmid (wavy line), into which was inserted a 1.85-kb PstI fragment (solid bar) permitting replication in Pseudomonas spp. (8). The open bar represents the structural gene sequence, with hacking on a cleaved signal sequence. The transcriptional start site is located at 658 bp, 88 bp upstream of the initiating codon.

ate, disodium carbenicillin, kanamycin (Sigma Chemical Co.), or FeSO₄·7H₂O was added to concentrations of 300 µg/ml, 50 µg/ml, or 0.1 mM, respectively. L broth (16) was used for some growth cultures. Restriction enzymes and Bal31 nuclease were obtained from Bethesda Research Laboratories, Inc.

**Methods.** Large-scale preparation of plasmid DNA was done as described by Birnboim and Doly (3). Isolation of plasmid DNA from transformant clones for screening procedures was done by the alkaline method (17). Restriction enzyme cleavage, ligation, and transformation of E. coli HB101 and P. aeruginosa MAM4 were carried out as described previously (20). E. coli AA102 was used for transformation of ligated DNA, essentially by the method of Hanahan (10). The recessed 3' ends of double-stranded DNA were filled in by the Klenow fragment of E. coli DNA polymerase I as described before (17). PvuI sites were converted to EcoRI sites with the hexamer TCGAAT (Pharmacia), based on the technique of Barany (2). Bal31 digestion followed the procedure described before (17), with 1 U of Bal31 nuclease per 16 µg of DNA in 100 µl at 30°C. EcoRI linker ligation was carried out with 1 µg of blunt-ended DNA, 3 µg of EcoRI linker (GGAATTCC; New England BioLabs), and 5 U of T4 DNA ligase in 10 µl and incubating at 7°C for 1 h and overnight at 12°C. The Km' cassette was excised as a 1.3-kb EcoRI fragment from pUC4-KISS and was incorporated by ligating approximately equal weights of cassette and EcoRI-cleaved plasmid DNA.

Exotoxin A synthesis and secretion were detected by using the Elek immunodiffusion test (5) for the rapid screening of clones grown on TSBD agar. A radioimmunoassay (12) was used for more quantitative measurements of exotoxin A in supernatants of MAM4 cultures after growth at 32°C in deferrated TSBD for 20 h. Dilutions of a purified exotoxin A eluted, dephosphorylated, [γ-32P]ATP end-labeled, and sequenced as described by Maxam and Gilbert (18).

**DNA sequencing.** After initial digestion by EcoRI and KpnI of 5' deletion mutants or by PstI and EcoRI of 3' deletion mutants, the digests were incubated with RNase I and then separated by polyacrylamide gel electrophoresis (PAGE) on 5% polyacrylamide gels. The fragments were then electrophoresed, dephosphorylated, [γ-32P]ATP end-labeled, and sequenced as described by Maxam and Gilbert (18).

**Plasmid constructions.** pRC362ΔE was constructed from pRC362 (Fig. 1) by removing the EcoRI site (nucleotide 2760) at the 3' terminus of the cloned exotoxin A gene (8) by EcoRI cleavage, followed by fill-in of recessed ends by DNA polymerase I (large fragment) and ligation of resulting blunt-end DNA by T4 DNA ligase. The ligation products were transformed into E. coli HB101, selecting carbenicillin-resistant (Cb') clones. The plasmid DNA from a clone that was shown to be insensitive to cleavage by EcoRI was designated pRC362ΔE.

pRC362(551E) was derived by subjecting pRC362ΔE DNA to partial cleavage by PvuI (sites at nucleotides 551 and 1135 in the exotoxin segment and two sites in the vector). TCGAAT linker DNA (Pharmacia) was added, and the mixture was ligated. It was then cleaved with EcoRI, and a 1.3-kb Km' cassette excised by EcoRI from pUC4-KISS was added and ligated. Following transformation into E. coli AA102 and selection for Km' colonies, the plasmid DNA from several clones was isolated and cleaved with PstI and EcoRI. One clone that produced two fragments, one of approximately 550 bp and the other of 1.3 kb, was selected, and the Km' cassette was removed following EcoRI digestion, dilution, and religation. The result of these procedures was to insert an EcoRI recognition sequence, TCGAATTC GAAATTC, between nucleotides 551 and 552, to produce the plasmid designated pRC362(551E).

**Deletion by Bal31.** EcoRI-cleaved plasmid DNA was digested by Bal31 for appropriate times. The recessed ends of the deleted DNAs were filled in and ligated to the 8-bp EcoRI linker sequence GGAATTC. Plasmids with an attached EcoRI linker sequence were then selected by EcoRI digestion, followed by ligation of a Km' cassette with EcoRI termini, which permitted growth of transformed AA102 hosts on NA plus kanamycin. The plasmid DNA of Km' transformants was digested with EcoRI, diluted, and religated to remove the Km' cassette.

**Determination of deletion sizes of end-labeled fragments.** As...
FIG. 2. The top line (WT, wild type) shows the nucleotide sequence as established 5' to the toxA gene of *P. aeruginosa* PA103 (8, 9) when cloned in the plasmid pRC362 (8). The numbers above are nucleotides distant from the *PstI* site (at nucleotide +1). The transcription initiation site (Pex) is at nucleotide 658 (horizontal arrow) (8), and the initiating codon is at nucleotide 746 (9). The numbers under the bottom line show distances in nucleotides from Pex. 5' deletions of this sequence terminating 73 bp or distal to Pex (shown by horizontal arrow and +) expressed exotoxin A at the level of pRC362 transformed into the nontoxigenic *P. aeruginosa* recipient MAM4. 5' deletions terminating at 69 bp or proximal to Pex (shown as horizontal arrow and −) expressed exotoxin A at a level of 5% or less of the pRC362 level. The lines below (LS-1 to LS-16) show the corresponding nucleotide sequences in linker-scanning mutants in which the boxed sequence shown above the line has been substituted for the sequence of pRC362 shown boxed. In LS-1 to LS-12, an equal number of nucleotides are substituted. In LS-13 to LS-16, the numbers of nucleotides substituted in the mutant are unequal to those of pRC362 which were replaced. The numbers to the right under Exo show the level of expression determined by a radioimmunoassay of each mutant when transformed into MAM4 and grown in TSBD medium with about 1 μM Fe**2+** compared with that of the parental plasmid pRC362 in the same host and the same medium. The values shown are the mean and standard deviation for at least three separate assays from separate cultures or, in the case of pRC362, for six separate cultures assayed. (In TSBD medium with 0.1 mM Fe**2+**, expression of exotoxin A by all mutants and by pRC362 was between 1 and 3% of the level of pRC362 with 1 μM Fe**2+**.)

a rapid method for more precise measurement of deletion sizes than allowed by electrophoretic mobility of DNA fragments in polyacrylamide gels, the following technique was used. The size of each deletion was initially roughly estimated by the mobility of restriction fragments in 8% polyacrylamide gels. In mutants carrying deletions from a site 5' to the transcription initiation site, *EcoR*I-*BamHI* (nucleotide 765) cleavage fragments were used, or, in the
case of mutants carrying deletions from a site 3’ to the transcription initiation site, EcoRI-NorI (nucleotide 450) fragments were measured (Fig. 1). Two other deletion mutants were then chosen in which the deletions had been previously defined by DNA sequence analysis (18), one of which had a longer (ΔL) and the other a smaller (ΔS) deletion than the deletion (ΔU) under study. For 5’ deletions, the DNA of all three deletion mutants was cleaved with Mael (unique site at nucleotide 653 in the exotoxin A sequence), end-labeled with [α-32P]dATP by Klenow fragment, filled-in with [α-32P]dATP, and cleaved with EcoRI. The DNAs of ΔL, ΔS, and ΔU were then separated on a 10% polyacrylamide-urea gel in parallel with the G+A and T+C sequencing reactions of ΔL or ΔS to permit measurement (∆±1 bp) of the size of ΔU from the 3’ label to the 5’-cleaved EcoRI terminus. For 3’ deletions, a similar procedure used cleavage and end-labeling of the EcoRI site with [α-32P]dATP, followed by PvuI cleavage at nucleotide 551.

RESULTS

Construction of 5’ deletions. Deletions of nucleotide sequences located 5’ to the exotoxin A transcription initiation site (Pex) at nucleotide 658 were derived by Bal31 digestion of pRC362(551E) from nucleotide 551 of the 2,760-bp exotoxin sequence (Fig. 1). pRC362(551E) was first cleaved by EcoRI and digested by Bal31 for 0, 30, 60, and 90 s, followed by EcoRI linker ligation and selection by use of a Km’ cassette. Following removal of the Km’ cassette, the plasmid DNA of several clones transformed with each timed Bal31 digest was assayed for the approximate size of the deletion by cleavage with EcoRI and BamHI (nucleotide 765), and then the transformed mam4 hosts were screened for expression of exotoxin A by an Elek test (5). From these tests, it was established that 18 mutants carrying deletions which terminated approximately 70 bp or more upstream of Pex expressed exotoxin A (by production of a precipitin line in the agar), whereas nine mutants carrying deletions which terminated within about 70 bp or less of Pex did not express exotoxin A (data not shown). The 60-s Bal31 digest produced some deletions which expressed the toxin and others which did not and was used for further analysis. The size and location of deletions which terminated about 70 bp or less upstream of Pex were more accurately determined (±1 bp) in the 60-s digest by the end-labeling method (data not shown). All clones in which toxin was not expressed in an Elek test were found to have deletions terminating within 69 bp from Pex, and all clones which expressed toxin had deletions terminating 73 bp or more from Pex. All hosts that expressed exotoxin A showed no expression when 0.1 mM iron was added to the medium. From these deletion experiments, we conclude that a sequence 72 bp upstream of Pex is necessary and sufficient for the expression and regulation of exotoxin A.

Construction of 3’ deletions. A series of Bal31-generated 3’ deletions with EcoRI linker insertions were similarly constructed following KpnI cleavage (unique site at nucleotide 1130) of pRC362ΔE. The plasmid DNA of several Km’ transformant clones from each digestion time of 0, 55, 65, and 75 min was screened for size as described for 5’ deletion mutants, except that EcoRI and NotI (nucleotide 450) were used to roughly determine the location of the 3’ end of the deletion. The 65-min digest, which produced a number of deletions with 3’ termini within the 72-bp region upstream of Pex, was chosen for more accurate (∆±1 bp) determinations of the deletion sizes.

Construction and analysis of linker-scanning mutants. Following precise determination by nucleotide sequencing of the termini of a number of 5’ and 3’ deletion mutants which had EcoRI linkers inserted within a 75-bp region upstream of Pex, 16 derivatives of pRC362 were constructed in which we attempted to substitute the EcoRI linker sequence GGAATTC to an 8-bp sequence within the region 75 bp upstream of Pex. This was achieved by cleavage and ligation between one of each type of deletion mutant in which the 5’ end of one and the 3’ end of another were found to be terminated by an EcoRI linker sequence at the identical location. The PSrl-EcoRI segment of the 3’ deletion and the EcoRI-KpnI segment of the 5’ deletion were separated by PAGe on a 5% polyacrylamide gel, excised, and electroeluted (Fig. 1). In each construct, the large (6.2-kb) Psrl-KpnI vector segment of pRC362ΔE, similarly separated from a 0.6% ultrapure agarose gel, was added to the two segments and ligated, and the ligation mixture was transformed directly into E. coli HB101. The plasmid DNA from several Clb’ clones was isolated and screened for size by PSrl-BamHI cleavage (765 bp) and by PvuI-BamHI cleavage (214 bp). The nucleotide sequence of about 40 bp in each direction from the introduced EcoRI site was then established by the Maxam-Gilbert technique for each construct to accurately locate the linker substitutions, as shown in Fig. 2.

In 10 of the constructs, the GGAATTC sequence was substituted for an equivalent 8-bp sequence in the 72-bp control region. In three mutants it replaced seven bases (in LS-13 and LS-15) or nine bases (in LS-16), and in three other mutants, reiterations of part of the GGAATTC sequence were substituted for an equivalent number of bases (in LS-3 and LS-5) or for a smaller number of bases (in LS-14) (Fig. 2). Plasmid DNA from E. coli clones carrying these mutants was then transformed into P. aeruginosa MAM4 to assay for exotoxin A. Toxin expression of the 16 linker-substitution mutants was measured by radioimmunoassays of the supernatant of the MAM4 host carrying the plasmid, grown either in the presence or absence of iron, and were quantitated by using a pure exotoxin A standard. In low-iron medium, the supernatants from MAM4(pRC362), assayed following a 1:200 dilution, were found to contain 6.0 ± 1.0 μg of exotoxin A per OD600 unit, whereas those from poorly expressing mutants, e.g., MAM4(pRC362LS-1), contained only 0.16 ± 0.05 μg/OD600 unit. The amounts of exotoxin A relative to that synthesized by pRC362 in low-iron medium are shown in Fig. 2. The nucleotide sequences of the region 5’ to the transcription initiation site of the mutants are constructed and the level of their expression in the Pseudomonas host MAM4 shown in Fig. 2 are summarized in Fig. 3. In only four regions (in LS-1, -2, -3, and -12) of the sequence upstream of the transcriptional initiation site did the substitution of a linker for an equivalent number of nucleotides result in more than a twofold change in expression, and in all of these, a decrease to 5% or less of the control level resulted.

DISCUSSION

Comparisons between the initial and substituted nucleotide sequences upstream of the toxA gene (Fig. 2) show that one or more nucleotides in the −74 to −54 sequence are essential for normal expression (LS-1, -2, -3, and -12), as are other nucleotides in the −9 to −2 sequence (LS-12). However, most nucleotides located at −53 to −14 can be substituted, and those located upstream of −72 can be deleted, as observed in the original 5’ deletion Bal31 mutants, without

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otides and -6 single Pex A. exotoxin sequences centered binding the nucleotides -41
region is also

FIG. 3. Nucleotide sequence 5' to the toxA gene as shown in Fig.
2. Nucleotides in capital letters without underlines were not sub-
tuted, and those in lowercase letters could be substituted with little
(or no more than a twofold) effect on expression, whereas substitu-
tion of those in capital letters with underlines, which are located
within a single box as shown in Fig. 2, resulted in a decrease in
expression to 5% or less. Boxed regions represent approximate
locations of putative binding sites for RNA polymerase (centered at
-6 and -41) and for a transcriptional activator molecule (centered at
-63).

having any more than a twofold influence on expression. This
limits the essential distal upstream sequence to nucleotides between -72 and -54. Moreover, the insertion of a single nucleotide within the -66 to -60 sequence (LS-13), or the insertion of four nucleotides in the -53 to -39 sequence (LS-14), also reduced expression to less than 5%. However, the insertion of an extra nucleotide between -48 and -42 (LS-15) or the loss of one nucleotide between -32 and -24 (LS-16) had little effect (Fig. 3).

We earlier concluded that the 72-bp sequence upstream of Pex is adequate and sufficient to define the expression of exotoxin A. It seems reasonable to propose that the sequences centered at nucleotides -41 and -6 are involved in the binding of an RNA polymerase, in which case the nucleotides between -72 and -54 appear to be necessary for the binding of an accessory transcriptional factor. That a single accessory-binding site is present in this region may be inferred from the fact that insertion of a single nucleotide within this region (in LS-13) severely reduced expression. Moreover, the distance between the -72 to -54 region and the -41 region is also critical, as deduced from the effects of inserting four nucleotides (compare LS-5 with LS-14), from which it may be proposed that the binding of the accessory factor at the -72 to -54 region may permit its interaction with the RNA polymerase molecule which binds at the -45 to -2 region.

Substitution of nucleotides between -45 and -38 (in LS-5, -6, or -7) produced smaller (limited to approximately twofold) differences in expression, in contrast to the changes of nucleotides -37 to -10 (in LS-8, -9, -10, and -11), where differences in expression from that produced by the wild-
type sequence did not exceed the standard error. It is proposed that the binding of an activator molecule at the -72 to -54 region may be required for the subsequent binding of an RNA polymerase at the -45 to -38 and the -9 to -2 regions. It might then be suggested that the sequence at the -45 to -38 region proximal to the activator-binding site would be of less importance to the stability of RNA poly-
merase binding (or open complex formation) and can lead only to twofold up or down differences, whereas binding at the -9 to -2 site distal to the activator-binding site is critical, so that 30-fold reductions in expression result from substitutions in this region.

The identification of a region necessary for binding a factor in addition to RNA polymerase which is required for transcription is consistent with the findings of Galloway and colleagues (12, 23), who concluded that a positive activator gene, toxR, was required for the efficient expression of exotoxin A. The -72 to -54 region identified here may be the binding site for this activator gene product. It may be noteworthy that this site includes a direct repeat of the sequence AAATCC at -71 to -67 and -58 to -54. However, the direct repeat, CTCCCCG, located at -35 to -28 and -23 to -16, suggested by Wozniak et al. (23) to be the binding site for the toxR product, does not in fact appear to be involved in the regulation of exotoxin A expression, since any of the nucleotides in the direct repeat (except for that at
-22, which was not changed) can be substituted with little effect (less than twofold) on expression.

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